A Survey of Phytotoxic Microbial and Plant Metabolites as Potential Natural Products for Pest Management

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Dedicated to the memory of Prof. *Carlo Rosini*

Phytotoxic microbial metabolites produced by certain phytopathogenic fungi and bacteria, and a group of phytotoxic plant metabolites including Amaryllidacea alkaloids and some derivatives of these compounds were evaluated for algicide, bactericide, insecticide, fungicide, and herbicide activities in order to discover natural compounds for potential use in the management and control of several important agricultural and household structural pests. Among the various compounds evaluated: *i*) ophiobolin A was found to be the most promising for potential use as a selective algicide; *ii*) ungeremine was discovered to be bactericidal against certain species of fish pathogenic bacteria; *iii*) cycasin caused significant mortality in termites; *iv*) cavoxin, ophiobolin A, and sphaeropsidin A were most active towards species of plant pathogenic fungi; and *v*) lycorine and some of its analogues (1-*O*-acetyllycorine and lycorine chlorohydrate) were highly phytotoxic in the herbicide bioassay. Our results further demonstrated that plants and microbes can provide a diverse and natural source of compounds with potential use as pesticides.

1. Introduction. – During the early years of chemical pest management, natural products were some of the most commonly used pesticides. However, during the middle part of the last century, the pesticide industry began to introduce an increasing arsenal of synthetic pesticides, initially with compounds such as DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) and 2,4-D ((2,4-dichlorophenoxy)acetic acid). These synthetic chemicals frequently have low specificity and are poorly biodegradable, thereby causing their accumulation in water supplies and soil, and eventual bioaccumulation in non-target organisms, producing heavy environmental pollution, and/or creating problems for human and animal health. In the past, many companies were also pharmaceutical ones. Thus, a portion of the pesticide-discovery effort was based upon natural products as templates for new structures, because the pharmaceutical discovery has relied heavily on natural products as promising compounds. Most of this approach with pesticides involved the screening of microbial metabolites, while other organisms (*e.g.*, terrestrial plants, algae, marine organisms, *etc.*) have received less emphasis [1].

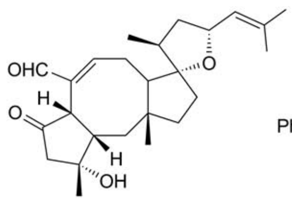
In recent years, a renewed interest in obtaining biologically active compounds from natural sources has emerged, notwithstanding the impressive progress of new methodologies such as combinational chemistry, high-throughput screening, and genetic engineering. Contributing to this worldwide attention towards pest-management products based upon natural products are their often low or absent toxicity towards non-target organisms, their near complete biodegradability, their availability from renewable sources, and, in many cases, their low-cost compared to those compounds obtained by complete chemical synthesis. A further impetus to the study of natural compounds for pest management is the fact that plants and microorganisms produce thousands of chemical substances, and only a minor fraction of species has been studied for this purpose. In addition, the study of bioactive secondary metabolites, traditionally carried out by chemists, has increasingly attracted the attention of pharmacologists, biologists, botanists, agronomists, geneticists, *etc.*, thereby stimulating cooperative work.

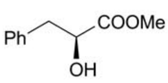
This report presents results and potential leads for pest management from a broad-spectrum evaluation of phytotoxic microbial and plant metabolites for their algicide, bactericide, fungicide, herbicide, and insecticide activities. The various pests included in this study were: *i*) an off-flavor compound-producing species of cyanobacteria (blue-green algae); *ii*) pathogenic bacteria which cause disease in pond-raised channel catfish (*Ictalurus punctatus*); *iii*) phytopathogenic species of fungi which cause disease in minor crops; and *iv*) the Formosan subterranean termite *Coptotermes formosanus*. Furthermore, the compounds were tested for herbicide activity using a standard bioassay. Laboratory evaluation *via* rapid bioassay of natural compounds is an imperative first stage in the discovery of novel compounds for use in pest management.

2. Results and Discussion. – *Compounds Tested.* The microbial metabolites (*Table 1*) used in this survey were purified from the culture filtrates of phytopathogenic fungi and bacteria, and all test compounds had previously been identified as phytotoxic as well as possessing some other interesting biological activities. The fungal test metabolites were brefeldin A, cavoxin, cyclopaldic acid, cytochalasin B (CYTO B), 21,22-dihydro-CYTO B, ophiobolin A, seiricuprolide, seiridin, and sphaeropsidin A. All of these compounds belong to different classes of natural products, including polyketide macrolides, chalcones, isobenzofuranones, perhydroisoindol-1-one macrolides, butenolides, diterpenes, and sesterterpenes. The only test metabolite derived from bacteria was the phytotoxic papuline, a methyl ester of a simple aromatic acid. The test metabolites from plants included cycasin, which is a glucoside of a methylazomethanol (MAM), while lycorine and ungeremine are Amaryllidacea alkaloids belonging to the pyrrolo[*d*,*e*]phenanthridine group. In particular, the ungeremine is a betaine, while clivonine hydrochloride was the salt obtained from clivonine, another Amaryllidacea alkaloid belonging to the lycorenine group.

Cytochalasin B was converted into its '21,22-dihydro' derivative by reduction of the α,β-unsaturated C=C bond with $NaBH_4$. By chemical transformation, four derivatives of fusicoccin (FC) were prepared. In particular, FC was first acetylated and then oxidized using *Jones*'s reagent to yield triacetyl-8-oxo-FC. The isomer of FC-deacetylaglycone and 19-trityl-12-oxo-8,9-isopropylidene-FC-aglycone were prepared by chemical modification of FC-deacetylaglycone, which was previously prepared from FC through deacetylation and oxidation, and β-elimination of the sugar moiety.

Six derivatives were obtained from lycorine. In particular, 1-*O*-acetyllycorine, 1,2-*O*,*O*-diacetyllycorine, and lycorin-2-one were prepared from lycorine by partial and


Ophiobolin A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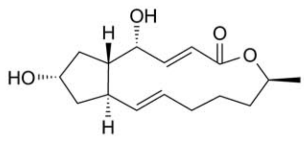
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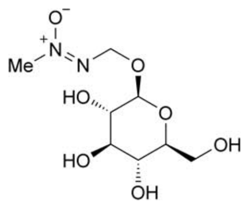
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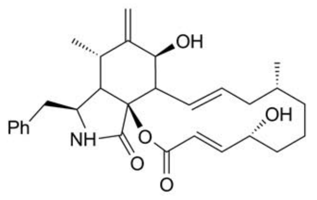
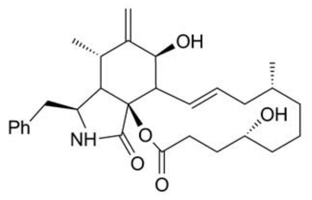
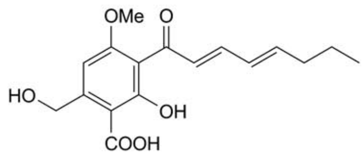
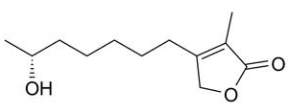
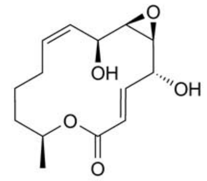
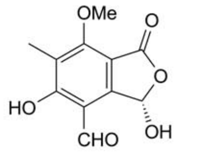

Cytochalasin B (CYTO B)

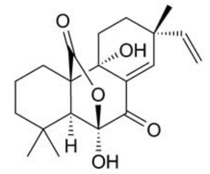

21,22-Dihydro-CYTO B

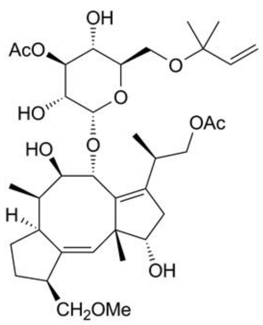

Cavoxin

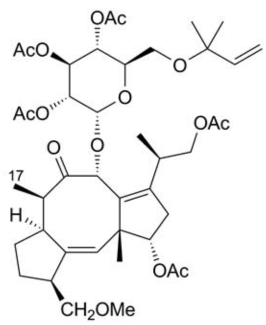

Seiridin

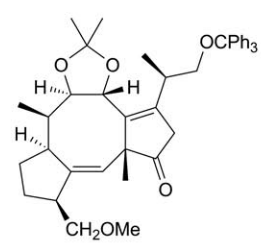

Seiricuprolide

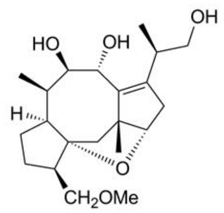

Cyclopaldic acid

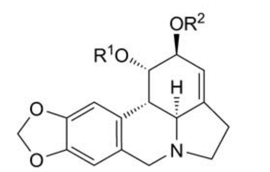

Sphaeropsidin A

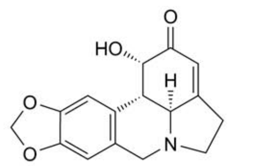

Fusicoccin (FC)


Triacetyl-8-oxo-FC


19-Trityl-12-oxo-8,9-isopropylidene FC-aglycone


Isomer of FC-deacetylaglycone


Lycorine $R^1 = R^2 = H$
1-*O*-Acetyllycorine $R^1 = Ac$, $R^2 = H$
1,2-*O*,*O*'-Diacetyllycorine $R^1 = R^2 = Ac$


Lycorin-2-one

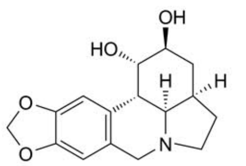

α-Dihydrolycorine

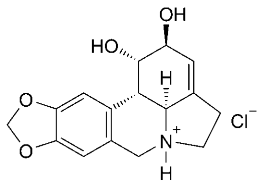
Table 1. *Bioactive Metabolites Evaluated for Algicidal, Bactericidal, Fungicidal, Herbicidal, and Insecticidal Activities*

Test compound[a])	Mol. wt.	Source and Ref.
1-*O*-Acetyllycorine	329	Derivative of lycorine [2]
Brefeldin A	280	*Alternaria zinniae* [3]
Cavoxin	320	*Phoma cava* [4]
Clivonine hydrochloride	366	*Clivia miniata* [2]
Cycasin	252	*Cycas revolute* spp. [5]
Cyclopaldic acid	238	*Seridium cupressi* [6]
Cytochalasin B (CYTO B)	479	*Pyrenophora semeniperda* [7]
1,2-Diacetyl-α-dihydrolycorine	373	Derivative of lycorine [2]
1,2-*O*,*O'*-Diacetyllycorine	371	Derivative of lycorine [2]
21,22-Dihydro-CYTO B	481	Derivative of CYTO B [8]
α-Dihydrolycorine	289	Derivative of lycorine [2]
Fusicoccin (FC)	680	*Fusicoccum amygdaly* [9]
Isomer of FC-deacetylaglycone[b])	366	Derivative from FC [10]
Lycorine	287	*Sternbergia lutea* [11]
Lycorine hydrochloride	323	Derivative of lycorine [11]
Lycorin-2-one	285	Derivative of lycorine [2]
N-Methyllycorine iodide	427	Derivative of lycorine [12]
Ophiobolin A	400	*Drechslera gigantea* [13]
Papuline	180	*Pseudomonas syringae* pv. *papulans* [14]
Seiricuprolide	268	*Seridium cupressi* [15]
Seiridin	346	*Seridium* spp. [16]
Sphaeropsidin A	346	*Sphaeropsis sapinea* f. sp. *cupressi* [17]
Triacetyl-8-oxo-FC	804	Derivative of FC [18]
Trityloxo[c])	646	Derivative of FC [19]
Ungeremine	265	*Pancratium maritimum* [20]

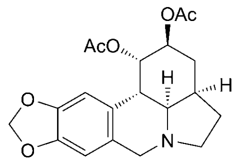
[a]) Abbreviation for the compounds are given in parentheses. [b]) Abbreviation for 1*H*-12-dehydro-2,12-epoxy-FC-deacetylaglycone. [c]) Abbreviation for 19-trityl-12-oxo-8,9-isopropylidene-FC-deacetylaglycone.

total acetylation or oxidation of the diol system of the *C*-ring, while α-dihydrolycorine and the corresponding diacetyl derivative were obtained by catalytic hydrogenation of lycorine, followed by acetylation. Finally, lycorine hydrochloride was obtained by treatment of the alkaloid with diluted HCl.

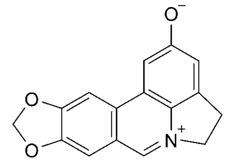
Algicidal Activity. In the southeastern United States pond-raised channel catfish (*Ictalurus punctatus*) industry, environmentally derived pre-harvest off-flavors create significant economic losses to producers due to delayed harvest. The most common types of these off-flavors are 'earthy' and 'musty' which are caused by the presence of the microbial metabolites geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) and 2-methylisoborneol (*exo*-1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol), respectively, in the fish flesh. In Mississippi, 'musty' off-flavor problems are more frequently encountered and can be attributed to the presence of the cyanobacterium *Planktothrix perornata* (Skuja) Anagnostidis & Komárek (previously designated as *Oscillatoria perornata* f. *attenuata* (Skuja) by *Schrader et al.* [21] and as *Oscillatoria* cf. *chalybea* by *Martin et al.* [22]) in catfish ponds [23][24]. Currently, the most common management approach



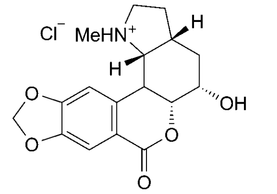
Lycorine hydrochloride



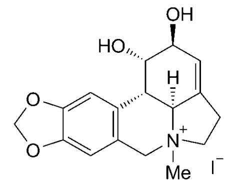
1,2-*O*,*O*'-Diacetyl-*α*-dihydrolycorine



Ungeremine



Clivonine hydrochloride



N-Methyllycorine iodide

used by catfish farmers is the application of the herbicide diuron (*N*'-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea) to the production ponds in order to reduce the abundance of *P. perornata* and subsequently levels of 2-methylisoborneol (MIB) in the pond water to allow depuration of MIB from the fish flesh. Diuron has been found to possess little selective toxicity towards *P. perornata* compared to other types of phytoplankton [25]. Due to this lack of selective toxicity and the potential for absorption of diuron into the catfish flesh, catfish farmers must carefully follow strict label-use guidelines for diuron which can result in repeated applications over several months (one application per 7-day period and up to nine applications per pond per year [26]). This management approach can still result in a delayed harvest, if proper protocol is not followed. The discovery of environmentally safe natural compounds that do not accumulate in catfish flesh and possess greater selective toxicity towards *P. perornata* would benefit the catfish production industry.

In this study, ophiobolin A was the most toxic toward *P. perornata* based upon the *LCIC* value of 10.0 μM (*Table 2*). Ophiobolin A was also selectively toxic toward *P. perornata* based upon comparison of *LOEC* and *LCIC* values with those obtained for *S. capricornutum*. The 96-h IC_{50} (50% inhibition concentration) value of ophiobolin A for *P. perornata* and *S. capricornutum* was 4.27 ± 2.49 and 11.36 ± 3.46 μM, respectively. Ophiobolin A is known to inhibit the Ca^{2+}-binding protein calmodulin which is ubiquitous in eukaryotes, and this protein is required for the function of Ca^{2+}-dependent activation of NAD kinase and in ATP-dependent Ca^{2+} uptake by microsomal vesicles in plants [27]. Although calmodulin has not been found in cyanobacteria, calmodulin-like proteins have been isolated from the cyanobacteria *Oscillatoria limnetica* [28], *Anabaena* spp. [29], and *Nostoc* sp. PCC 760 [30], and, therefore, ophiobolin A may also have a similar mode of action against cyanobacteria. Also in this study, brefeldin A was selectively toxic towards *S. capricornutum* (*LCIC* 1.0 μM). These results are likely due to the mode of action of brefeldin A identified by *Fujiwara et al.* [31], in which protein transport from the endoplasmic reticulum to the *Golgi* complex, two organelles not found in prokaryotes, is blocked.

Table 2. *Algicidal Activity by Evaluation of Bioactive Metabolites for Selective Toxicity toward* Planktothrix peronata

Test compound	*Planktothrix perornata*		*Selenastrum capricornutum*	
	LOEC[a]) [μM]	*LCIC*[b]) [μM]	*LOEC* [μM]	*LCIC* [μM]
1-*O*-Acetyllycorine	> 100.0	> 100.0	10.0	100.0
Brefeldin A	> 100.0	> 100.0	0.1	1.0
Cavoxin	100.0	100.0	> 100.0	> 100.0
Clivonine hydrochloride	> 100.0	> 100.0	> 100.0	> 100.0
Cycasin	> 100.0	> 100.0	1.0	> 100.0
Cyclopaldic acid	100.0	100.0	100.0	100.0
Cytochalasin B (CYTO B)	100.0	> 100.0	0.1	> 100.0
1,2-Diacetyl-α-dihydrolycorine	> 100.0	> 100.0	1.0	> 100.0
1,2-*O*,*O'*-Diacetyllycorine	100.0	100.0	10.0	100.0
21,22-Dihydro-CYTO B	10.0	100.0	10.0	100.0
α-Dihydrolycorine	nd[c])	nd	nd	nd
Fusicoccin (FC)	> 100.0	> 100.0	> 100.0	> 100.0
Isomer of FC-deacetylaglycone	> 100.0	> 100.0	> 100.0	> 100.0
Lycorine	> 100.0	> 100.0	10.0	100.0
Lycorine hydrochloride	> 100.0	> 100.0	0.1	100.0
Lycorin-2-one	> 100.0	> 100.0	> 100.0	> 100.0
N-Methyllycorine iodide	nd	nd	nd	nd
Ophiobolin A	10.0	10.0	0.1	100.0
Papuline	> 100.0	> 100.0	> 100.0	> 100.0
Seiricuprolide	> 100.0	> 100.0	> 100.0	> 100.0
Seiridin	> 100.0	> 100.0	0.1	> 100.0
Sphaeropsidin A	100.0	> 100.0	100.0	> 100.0
Triacetyl-8-oxo-FC	> 100.0	> 100.0	> 100.0	> 100.0
Trityloxo	> 100.0	> 100.0	> 100.0	> 100.0
Ungeremine	> 100.0	> 100.0	0.1	> 100.0

[a]) *LOEC* = Lowest observed-effect concentration. [b]) *LCIC* = Lowest complete-inhibition concentration. [c]) nd = Not determined due to insufficient test material.

Bactericide Assay Results. The bacterial diseases columnaris and enteric septicemia of catfish (ESC) cause some of the largest economic losses to producers of pond-raised channel catfish in the United States of America. Columnaris and ESC are caused by *Flavobacterium columnare* and *Edwardsiella ictaluri*, respectively. There are several management approaches available to producers including the application of medicated feeds (*e.g.*, *Romet*® *30* for ESC), attenuated vaccines [32], and nonantibiotic therapeutants such as 35% *Perox-Aid*® for external columnaris. However, *Perox-Aid*® is not for use in earthen ponds with no water exchange. Additional therapeutants such as copper sulfate pentahydrate ($CuSO_4 \cdot 5\,H_2O$) and potassium permanganate ($KMnO_4$) have been cited as potential treatments for columnaris [33]. However, the efficacy of these therapeutants can be adversely impacted by certain water quality variables, and these compounds must also be applied carefully due to their broad-spectrum toxicity towards non-target organisms (*e.g.*, channel catfish) [34].

In this study, ungeremine was the only test compound with toxicity against *E. ictaluri* at the concentrations evaluated (*Table 3*). The 24-h IC_{50} and *MIC* values were

Table 3. *Antibacterial Activity of Test Metabolites toward* Edwardsiella ictaluri

Test compound	24-h IC_{50}[a])	*MIC*[b])	24-h IC_{50}		*MIC*	
			RDCF[c])	RDCO[d])	RDCF	RDCO
1-*O*-Acetyllycorine	>33	>33	>204	>385	>100	>111
Brefeldin A	>280	>280	>2041	>3846	>1000	>1111
Cavoxin	>320	>320	>2041	>3846	>1000	>1111
Clivonine hydrochloride	>37	>37	>204	>385	>100	>111
Cycasin	>252	252	>2041	>3846	1000	1111
Cyclopaldic acid	>238	>238	>2041	>3846	>1000	>1111
Cytochalasin B (CYTO B)	>479	>479	>2041	>3846	>1000	>1111
1,2-Diacetyl-α-dihydrolycorine	>37	>37	>204	>385	>100	>111
1,2-*O*,*O′*-Diacetyllycorine	>37	>37	>204	>385	>100	>111
21,22-Dihydro-CYTO B	>481	>481	>2041	>3846	>1000	>1111
α-Dihydrolycorine	nd[e])	nd	nd	nd	nd	nd
Fusicoccin (FC)	>680	>680	>2041	>3846	>1000	>1111
Isomer of FC-deacetylaglycone	>366	>366	>2041	>3846	>1000	>1111
Lycorine	>287	>287	>2041	>3846	>1000	>1111
Lycorine hydrochloride	>32	>32	>204	>385	>100	>111
Lycorin-2-one	>26	>26	>204	>385	>100	>111
N-Methyllycorine iodide	nd	nd	nd	nd	nd	nd
Ophiobolin A	>400	400	>2041	>3846	1000	>1111
Papuline	>180	>180	>2041	>3846	>1000	>1111
Seiricuprolide	>268	268	>2041	>3846	1000	>1111
Seiridin	>212	212	>2041	>3846	1000	>1111
Sphaeropsidin A	>346	35	>2041	>3846	100	111
Triacetyl-8-oxo-FC	>804	>804	>2041	>3846	>1000	>1111
Trityloxo	>646	>646	>2041	>3846	>1000	>1111
Ungeremine	58	3	449	846	10	11

[a]) 24-h 50% Inhibition concentration in mg/l. [b]) Minimum inhibitory concentration in mg/l. [c]) Relative to drug control florfenicol. [d]) Relative to drug control oxytetracycline. [e]) nd = Not determined.

58 ± 0 and 3 ± 0 mg/l, respectively. The following compounds were the most toxic towards both isolates of *F. columnare* based upon the 24-h IC_{50} and *MIC* values: 1-*O*-acetyllycorine, 1,2-*O*,*O′*-diacetyllycorine, 21,22-dihydrocytochalasin B, lycorine, ophiobolin A, sphaeropsidin A, and ungeremine (*Tables 4* and *5*). Ungeremine was the most toxic based upon 24-h IC_{50} values (0.8 ± 0 and 0.9 ± 0.2 mg/l for *F. columnare ALM-00-173* and *F. columnare BioMed*, resp.) and 24-h IC_{50} RDCF and RDCO values (see *Tables 4* and *5*). Based upon *MIC* values, ungeremine (*MIC* 3 ± 0 mg/l) was also among the most toxic compounds towards both isolates as well as 1-*O*-acetyllycorine and 1,2-*O*,*O′*-diacetyllycorine. However, 1,2-*O*,*O′*-diacetyllycorine was less toxic towards *F. columnare BioMed* (*MIC* 37 mg/l) than *F. columnare ALM-00-173* (*MIC* 4 mg/l). Genomovar II isolates of *F. columnare* (*e.g.*, *ALM-00-173*) have been reported to be more pathogenic to immunocompetent channel catfish [35]. Overall, ungeremine collectively had the lowest 24-h IC_{50}, *MIC*, 24-h IC_{50} RDCF and RDCO, and *MIC* RDCF and RDCO values of any of the most active test compounds towards both *F. columnare* isolates (see *Tables 4* and *5*). Although ungeremine was the most active test compound against the *Gram*-negative bacteria used in this study, the *Gram*-positive

Table 4. *Antibacterial Activity of Test Metabolites toward* Flavobacterium columnare (*ALM-00-173*)

Test compound	24-h IC_{50} [a])	*MIC* [b])	24-h IC_{50}		*MIC*	
			RDCF [c])	RDCO [d])	RDCF	RDCO
1-*O*-Acetyllycorine	10	3	15	14	10	11
Brefeldin A	>280	280	>518	>493	1000	1075
Cavoxin	>320	>320	>518	>492	>1000	>1075
Clivonine hydrochloride	>37	>37	>52	>49	>100	>108
Cycasin	>252	>252	>518	>493	1000	>1075
Cyclopaldic acid	81	238	176	168	1000	1075
Cytochalasin B (CYTO B)	139	48	150	143	100	108
1,2-Diacetyl-α-dihydrolycorine	>37	>37	>52	>49	>100	>108
1,2-*O*,*O*′-Diacetyllycorine	2	4	3	3	10	11
21,22-Dihydro-CYTO B	14	48	16	15	100	108
α-Dihydrolycorine	nd [e])	nd	nd	nd	nd	nd
Fusicoccin (FC)	>680	68	>518	>493	100	108
Isomer of FC-deacetylaglycone	>366	366	>518	>493	1000	1075
Lycorine	27	29	49	47	100	108
Lycorine hydrochloride	>32	3	>52	>49	100	108
Lycorin-2-one	>26	>26	>52	>49	>100	>108
N-Methyllycorine iodide	nd	nd	nd	nd	nd	nd
Ophiobolin A	12	40	16	15	100	108
Papuline	>180	>180	>518	>493	>1000	>1075
Seiricuprolide	>268	>268	>518	>493	>1000	>1075
Seiridin	>212	>212	>518	>493	>1000	>1075
Sphaeropsidin A	12	35	18	17	100	108
Triacetyl-8-oxo-FC	265	80	171	163	100	108
Trityloxo	>646	646	>518	>493	1000	1075
Ungeremine	0.8	3	2	2	10	11

[a]) 24-h 50% Inhibition concentration in mg/l. [b]) Minimum inhibitory concentration in mg/l. [c]) Relative to drug control florfenicol. [d]) Relative to drug control oxytetracycline. [e]) nd = Not determined.

bacterium *Staphylococcus aureus* was inhibited by ungeremine at only the highest test concentration (*MIC* 265 ± 0 mg/l; *Table 6*). The only two test compounds with toxicity against *S. aureus* at the concentrations evaluated were 21,22-dihydrocytochalasin B and sphaeropsidin A. For 21,22-dihydrocytochalasin B, the 24-h IC_{50} and *MIC* values were 154 and 48 mg/l, respectively, while sphaeropsidin A had 24-h IC_{50} and *MIC* values of 14 and 35 mg/l, respectively (*Table 6*).

Ungeremine has been isolated from the extracts of a variety of plant species, including *Ungernia minor* [36], *Crinum americanum* [37], *C. asiaticum* [38], *Zephyranthes flava* [39], and *Pancratium maritimum* [20], and also found in the lubber grasshopper *Brachystola magna*, most likely from the direct or indirect (*e.g.*, consumption of other grasshoppers) ingestion of plant material [40]. Previous research evaluating the toxicity of ungeremine against ten different bacterial isolates (both *Gram*-positive and *Gram*-negative) including *S. aureus* identified the *MIC* value to be between 25–50 mg/l [41]. In our bioassay, the *MIC* value was 3 mg/l for the three *Gram*-negative bacteria used and a *MIC* value of 265 mg/l for *S. aureus*. The difference in the results may be due to the manner in which the bioassay was performed; we used

Table 5. *Antibacterial Activity of Test Metabolites toward* Flavobacterium columnare (*BioMed*)

Test compound	24-h IC_{50} [a])	*MIC* [b])	24-h IC_{50}		*MIC*	
			RDCF [c])	RDCO [d])	RDCF	RDCO
1-*O*-Acetyllycorine	9	3	12	16	10	11
Brefeldin A	> 280	280	> 424	> 588	1000	1075
Cavoxin	> 320	> 320	> 424	> 588	> 1000	> 1075
Clivonine hydrochloride	> 37	> 37	> 42	> 59	> 100	> 108
Cycasin	> 252	> 252	> 424	> 588	> 1000	> 1075
Cyclopaldic acid	48	24	85	118	100	108
Cytochalasin B (CYTO B)	125	48	110	153	100	108
1,2-Diacetyl-α-dihydrolycorine	> 37	> 37	> 42	> 59	> 100	> 108
1,2-*O*,*O*′-Diacetyllycorine	10	37	11	15	100	108
21,22-Dihydro-CYTO B	13	48	12	16	100	108
α-Dihydrolycorine	nd [e])	nd	nd	nd	nd	nd
Fusicoccin (FC)	> 680	680	> 424	> 588	1000	1075
Isomer of FC-deacetylaglycone	> 366	366	> 424	> 588	1000	1075
Lycorine	55	29	81	59	100	108
Lycorine hydrochloride	> 32	32	> 42	> 59	100	108
Lycorin-2-one	> 26	> 26	> 42	> 59	> 100	> 108
N-Methyllycorine iodide	nd	nd	nd	nd	nd	nd
Ophiobolin A	14	40	14	20	100	108
Papuline	> 180	> 180	> 424	> 588	> 1000	> 1075
Seiricuprolide	> 268	> 268	> 424	> 588	> 1000	> 1075
Seiridin	> 212	212	> 424	> 588	1000	1075
Sphaeropsidin A	11	35	13	18	100	108
Triacetyl-8-oxo-FC	> 804	80	> 424	> 588	100	108
Trityloxo	258	65	169	235	100	108
Ungeremine	0.9	3	2	2	10	11

[a]) 24-h 50% Inhibition concentration in mg/l. [b]) Minimum inhibitory concentration in mg/l. [c]) Relative to drug control florfenicol. [d]) Relative to drug control oxytetracycline. [e]) nd = Not determined.

broth (liquid) media to conduct our bioassay, while *Ghosal et al.* [41] used nutrient agar (solid) media. The antibacterial activity of ungeremine has not been elucidated yet. Interestingly, lycorine, which was converted by microbial transformation *via Pseudomonas* sp. to ungeremine [42], was less toxic than ungeremine towards test bacteria in our study and in the study by *Ghosal et al.* [41]. The formulation of ungeremine to impart water solubility needs to be addressed, also taking into account that it is a betaine compound, before pursuing efficacy studies to further determine its potential use as a therapeutant in catfish aquaculture.

Herbicide/Phytotoxicity Results. Most of the compounds were not highly phytotoxic (*Table 7*). However, lycorine and some of its analogs (1-*O*-acetyllycorine and lycorine hydrochloride) were highly phytotoxic. *A. stolonifera* was more affected than lettuce by these compounds. Lycorine has been isolated as a potential allelochemical from *Lycoris radiata* Herb. [43]. It was also reported by *Evidente* and *Motta* [44] to be phytotoxic. This is not surprising because it is considered to be generally cytotoxic. Brefeldin A was moderately phytotoxic, and it has been previously reported to be so [3]. Compounds with little or no activity in our bioassays include cavoxin, triacetyl-8-oxo-FC (keto),

Table 6. *Antibacterial Activity of Test Metabolites toward* Staphylococcus aureus

Test compound	24-h IC_{50} [a])	*MIC* [b])	24-h IC_{50}		*MIC*	
			RDCF [c])	RDCO [d])	RDCF	RDCO
1-*O*-Acetyllycorine	> 33	> 33	> 41	> 476	> 100	> 1111
Brefeldin A	> 280	> 280	> 407	> 4762	> 1000	> 11111
Cavoxin	> 320	> 320	> 407	> 4762	> 1000	> 11111
Clivonine hydrochloride	> 37	> 37	> 41	> 476	> 100	> 1111
Cycasin	> 252	> 252	> 407	> 4762	> 1000	> 11111
Cyclopaldic acid	> 238	> 238	> 407	> 4762	> 1000	> 11111
Cytochalasin B (CYTO B)	> 479	> 479	> 407	> 4762	> 1000	> 11111
1,2-Diacetyl-α-dihydrolycorine	> 37	> 37	> 41	> 476	> 100	> 1111
1,2-*O*,*O*′-Diacetyllycorine	> 37	> 37	> 41	> 476	> 100	> 1111
21,22-Dihydro-CYTO B	154	48	130	1524	100	1111
α-Dihydrolycorine	nd [e])	nd	nd	nd	nd	nd
Fusicoccin (FC)	> 680	> 680	> 407	> 4762	> 1000	> 11111
Isomer of FC-deacetylaglycone	> 366	> 366	> 407	> 4762	> 1000	> 11111
Lycorine	> 287	> 287	> 407	> 4762	> 1000	> 11111
Lycorine hydrochloride	> 32	> 32	> 41	> 476	> 100	> 1111
Lycorin-2-one	> 26	> 26	> 41	> 476	> 100	> 1111
N-Methyllycorine iodide	nd	nd	nd	nd	nd	nd
Ophiobolin A	120	400	122	1429	1000	11111
Papuline	> 180	> 180	> 407	> 4762	> 1000	> 11111
Seiricuprolide	> 268	> 268	> 407	> 4762	> 1000	> 11111
Seiridin	> 212	> 212	> 407	> 4762	> 1000	> 11111
Sphaeropsidin A	14	35	16	190	100	1111
Triacetyl-8-oxo-FC	> 804	80	> 407	> 4762	100	1111
Trityloxo	> 646	> 646	> 407	> 4762	> 1000	> 11111
Ungeremine	> 265	265	> 407	> 4762	1000	11111

[a]) 24-h 50% Inhibition concentration in mg/l. [b]) Minimum inhibitory concentration in mg/l. [c]) Relative to drug control florfenicol. [d]) Relative to drug control oxytetracycline. [e]) nd = Not determined.

sphaeropsidin A, and sericuprolide; these compounds have been reported to have phytotoxicity in other bioassays [4][15][17][18]. As we found, others have also determined papuline [14], fusicoccin [9], and seiridin [16] to have moderate phytotoxicity in other systems. However, we found cytochalasin B, 21,22-dihydrocytochalasin B, and ophiobolin A to have weak activity, while others found these compounds to be phytotoxic in other bioassays [7][13][45].

Termite Bioassay Results. Several of the compounds included in this study were suspected of having potential activity against insects, and, therefore, these compounds were evaluated against the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. In a 21-day feeding bioassay, *C. formosanus* were placed on treated filter paper at 2% (*w*/*w*) or an untreated control, and termite mortality was evaluated daily. Of the 25 compounds evaluated, only cycasin demonstrated activity (*Table 8*). By day 3, a significant percent mortality was observed (7.5 ± 6.5%), and 100% mortality was obtained by day 9. The filter paper consumed was also determined to be 9.1 ± 3.6 mg, which was significantly lower than untreated control of 44.6 ± 0 mg. None of the other compounds evaluated were active.

Table 7. *Herbicidal Activity of Natural Compounds towards Lettuce and* Agrostis *after 7 Days of Exposure*

Compound	Test concentration [mg/ml]	Ranking[a]) Lettuce	*Agrostis*
1-*O*-Acetyllycorine	1.0	5	5
	0.1	0	4
Brefeldin A	1.0	3	4
	0.1	2	3
Cavoxin	1.0	1	0
	0.1	0	0
Clivonine hydrochloride	1.0	0	1
	0.1	0	0
Cycasin	1.0	0	0
	0.1	0	0
Cytochalasin B (CYTO B)	1.0	2	2
	0.1	0	0
1,2-Diacetyl-α-dihydrolycorine	1.0	1	3
	0.1	0	0
1,2-*O*,*O*′-Diacetyllycorine	1.0	4	4
	0.1	1	0
21,22-Dihydro-CYTO B	1.0	1	2
	0.1	0	0
α-Dihydrolycorine[b])	1.0	0	1
Fusicoccin (FC)	1.0	3	1
	0.1	1	0
Isomer of FC-deacetylaglycone	1.0	0	1
	0.1	0	0
Lycorine	1.0	5	5
	0.1	3	4
Lycorine hydrochloride	1.0	4	5
	0.1	3	5
Lycorin-2-one	1.0	0	0
	0.1	0	0
N-Methyllycorine iodide	1.0	1	3
	0.1	0	1
Ophiobolin A	1.0	2	4
	0.1	0	0
Papuline	1.0	4	4
	0.1	0	0
Seiricuprolide	1.0	1	0
	0.1	0	0
Seiridin	1.0	0	4
	0.1	0	0
Sphaeropsidin A[b])	1.0	0	0
Triacetyl-8-oxo-FC	1.0	1	0
	0.1	0	0
Trityloxo	1.0	0	0
	0.1	0	0
Ungeremine	1.0	1	1
	0.1	0	0

[a]) 0 = No effect; 5 = complete growth inhibition. [b]) Insufficient sample material for testing two concentrations.

Table 8. *Antitermite Activity towards* Coptotermes formosanus SHIRAKI *on Filter Paper Treated with 2% of Test Compound*

Compound	Mortality [%] (mean ± S.D.)[a])[b])						
	Days						
	1	3	6	9	15	19	21
1-*O*-Acetyllycorine	0A	0A	0A	0A	0A	0A	0A
Brefeldin A	0A	0A	0A	0A	0A	0A	0A
Cavoxin	0A	0A	0A	0A	0A	0A	0A
Clivonine hydrochloride	0A	0A	0A	0A	0A	0A	0A
Cycasin	0A	7.5 ± 6.5B	87.5 ± 25.0B	100.0 ± 0B	100.0 ± 0B	100.0 ± 0B	100.0 ± 0B
Cyclopaldic acid	0A	0A	0A	0A	0A	0A	0A
Cytochalasin B (CYTO B)	0A	0A	0A	0A	0A	0A	0A
1,2-Diacetyl-α-dihydrolycorine	0A	0A	0A	0A	0A	0A	0A
1,2-*O*,*O*′-Diacetyllycorine	0A	0A	0A	0A	0A	0A	0A
21,22-Dihydro-CYTO B	0A	0A	0A	0A	0A	0A	0A
α-Dihydrolycorine	0A	0A	0A	0A	0A	0A	0A
Fusicoccin (FC)	0A	0A	0A	0A	0A	0A	0A
Isomer of FC-deacetylaglycone	0A	0A	0A	0A	0A	0A	0A
Lycorine	0A	0A	0A	0A	0A	0A	0A
Lycorine hydrochloride	0A	0A	0A	0A	0A	0A	0A
Lycorin-2-one	0A	0A	0A	0A	0A	0A	0A
N-Methyllycorine iodide	0A	0A	0A	0A	0A	0A	0A
Ophiobolin A	0A	0A	0A	0A	0A	0A	0A
Papuline	0A	0A	0A	0A	0A	0A	0A
Seiricuprolide	0A	0A	0A	0A	0A	0A	0A
Seiridin	0A	0A	0A	0A	0A	0A	0A
Sphaeropsidin A	0A	0A	0A	0A	0A	0A	0A
Triacetyl-8-oxo-FC	0A	0A	0A	0A	0A	0A	0A
Trityloxo	0A	0A	0A	0A	0A	0A	0A
Ungeremine	0A	0A	0A	0A	0A	0A	0A
Untreated	0A	0A	0A	0A	0A	0A	0A

[a]) 20 Workers ($\geq$ 3rd instar)/4 replications. S.D. = Standard deviation. [b]) Means within a column/treatment with the same letter are not significantly different, LSD (= Least-Significant Difference): ($P < 0.05$).

Cycasin was further evaluated in a dose – response manner in the same bioassay against *C. formosanus* at concentrations of 1 to 0.05%. Complete mortality was not observed at any of these concentrations out to 21 d; however, by day 15 at 1% *w*/*w*, 91.3 ± 10.3% mortality was observed (*Table 9*). The filter paper consumed was significantly reduced at concentrations of both 1 and 0.5% with values of 6.6 ± 9.9 and 7.8 ± 15.4 mg, respectively, and relative to 44.6 ± 0 mg for untreated control (*Table 10*).

Fungicide Bioassay Results. Preliminary evaluation of microbial metabolites at millimolar concentrations using direct bioautography with two *Colletotrichum* species as the indicator species indicated that cavoxin, ophiobolin A, sphaeropsidin A, and cyclopaldic acid were promising compounds (*Table 11*). More in-depth dose-response studies at micromolar concentrations showed that the three most active compounds were cavoxin, ophiobolin A, and sphaeropsidin A. Antifungal activity at lower

Table 9. *Antitermite Activity towards* Coptotermes formosanus Shiraki *on Filter Paper Treated with Cycasin*

Cycasin concentration	Mortality [%] (mean ± S.D.)[a])[b])						
	Days						
	1	3	6	9	15	19	21
1.0%	0A	0A	30.0 ± 32.4	67.5 ± 25.3	91.3 ± 10.3A	91.3 ± 10.3	91.3 ± 10.3A
0.5%	0A	0A	13.8 ± 17.0AB	30.0 ± 44.B	67.5 ± 39.5A	68.8 ± 37.1	71.3 ± 32.2A
0.1%	0A	0A	0B	0B	1.3 ± 2.5B	1.3 ± 2.5B	1.3 ± 2.5B
0.05%	0A	0A	1.3 ± 2.5B	1.3 ± 2.5B	1.3 ± 2.5B	1.3 ± 2.5B	1.3 ± 2.5B
Untreated	0A	0A	0B	0B	0B	0B	0B

[a]) 20 Workers ($\geq$ 3rd instar)/4 replications. S.D. = Standard deviation. [b]) Means within a column/treatment with the same letter are not significantly different, LSD: $P < 0.05$.

Table 10. *Antitermite Activity Determined by Filter Paper Consumed* (mg: mean ± S.D.) *by* Coptotermes formosanus Shiraki *Following Treatment with Cycasin*

Compound	Solvent	Sample [% *w/w*]	Consumption[a])[b]) [mg]
Cycasin	MeOH	1.0	6.6 ± 9.9
Cycasin	MeOH	0.5	7.8 ± 15.4
Cycasin	MeOH	0.1	44.6 ± 0B
Cycasin	MeOH	0.05	44.6 ± 0B
Untreated	–	–	44.6 ± 0B

[a]) 20 Workers ($\geq$ 3rd instar)/4 replications. S.D. = Standard deviation. [b]) Means within a column/treatment with the same letter are not significantly different, LSD: $P < 0.05$.

concentrations was also specific to *Phomopsis*, and little activity was observed against the other test fungi. Cyclopaldic acid was less active than the captan standard.

At the highest concentration of 30 μM, cavoxin demonstrated 99.9%, ophiobolin A 97.9%, and sphaeropsidin A 72.5% fungal growth inhibition, and these compounds were more active than captan (59.6%) standard (*Fig. 1*). At 3.0 μM, cavoxin with 77.7% and ophiobolin A with 58.7% fungal growth inhibition were slightly more active than captan (56.6%) against *Phomopsis obscurans*. Similar antifungal activity was observed for *P. viticola*, where cavoxin produced 100% growth inhibition, ophiobolin A 76.2%, and sphaeropsidin A 69.5%, and they were more active than the captan (46.16%) standard at 30 μM (*Fig. 2*). At 3.0 μM, ophiobolin A showed 70.6% growth inhibition and sphaeropsidin A 58.5%, and both compounds were more active than captan (22.4%). These compounds showed potential for further development for control of *Phomopsis* leaf and stem diseases with limited activity against the other test fungi (*Botrytis cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *Fusarium oxysporum*). This apparent selective activity towards *Phomopsis* indicates that these compounds may have relatively limited fungicidal activity against non-target fungi. *Phomopsis* cane and leaf spot (*P. viticola*) cause serious economic losses to the vine grape industry in USA and Europe, while *P. obscurans* causes *Phomopsis* leaf blight and fruit rot of strawberry.

Table 11. *Fungicidal Activity Determined by Mean Zone Diameter from Direct Bioautography of Test Compounds against* Colletotrichum fragariae *and* Colletotrichum acutatum

Compound[c])	Diameter [cm]					
	Cf 63[a])		Ca Goff[b])			
	4 µl	8 µl	4 µl	S.D.	8 µl	S.D.
Brefeldin A	0	0	0	0	0	0
Cavoxin	0.9	1	1.02	0.44	1.30	0.30
Cycasin	0	0	0	0	0	0
Cyclopaldic acid	0	0	0.35	0.61	0.40	0.69
Cytochalasin B (CYTO B)	0	0	0	0	0	0
21,22-Dihydro-CYTO B	0	0	0	0	0	0
Ophiobolin A	0	0	1.13	0.78	1.62	0.73
Papuline	0	0	0	0	0	0
Seiricuprolide	0	0	0	0	0	0
Seiridin	0	0	0	0	0	0
Sphaeropsidin A	0	0	0.27	0.46	0.80	1.39
Captan[d])	0.18		0.19	0		
Azoxystrobin[d])	0.13		0.14	0		
Benomyl[d])	0.12		0.12	0		

[a]) Cf 63 = *C. fragariae* isolate # 63; data only reported once due to contamination issues, *i.e.*, no standard deviation. [b]) Ca Goff = *Colletotrichum acutatum* isolate. S.D. = Standard deviation; standard deviation reported over three sampling times. [c]) Working solutions of each compound (2 mM in EtOH) were prepared, and then 4 µl or 8 µl of each respective solution were applied to the TLC plate. [d]) The commercial fungicide standards captan, azoxystrobin, and benomyl were run only at the 4-µl volume of compound addition.

Conclusions. – The results of biological assays, including antibacterial, algicidal, herbicidal, antitermite, and fungicidal activities demonstrate that plants and microbes can provide a diverse and natural source of compounds with potential use as pesticides. Among the various compounds evaluated, ophiobolin A was found to be the most promising for potential use as a selective algicide; ungeremine was determined to be bactericidal against certain species of fish pathogenic bacteria; cycasin caused significant mortality in termites; cavoxin, ophiobolin A, and sphaeropsidin A were the most active compounds towards species of plant pathogenic fungi; and lycorine and some of its analogs (1-*O*-acetyllycorine and lycorine chlorohydrate) were highly phytotoxic in the herbicide bioassay.

Experimental Part

Sources of Test Compounds: Microbial Metabolites and Their Derivatives. Brefeldin [3], cyclopaldic acid [6], seiricuprolide [15], cytochalasin B [7], ophiobolin A [13], sphaeropsidin A [17], and cavoxin [4] were purified as pale yellow and white needles from the culture filtrates of *Alternaria zinniae*, *Seridium cupressi*, *Pyrenophora semeniperda*, *Drechslera gigantea*, and *Phoma cava* as previously reported. Papuline [14] and seiridin [16] were purified as pure oils from the culture filtrates of *Pseudomonas syringae* pv. *papulans* and *Seiridium cardinale* , resp. The '21,22-dihydro' derivative of cytochalasin B was prepared from cytochalasin B by $NaBH_4$ reduction as reported in [8].

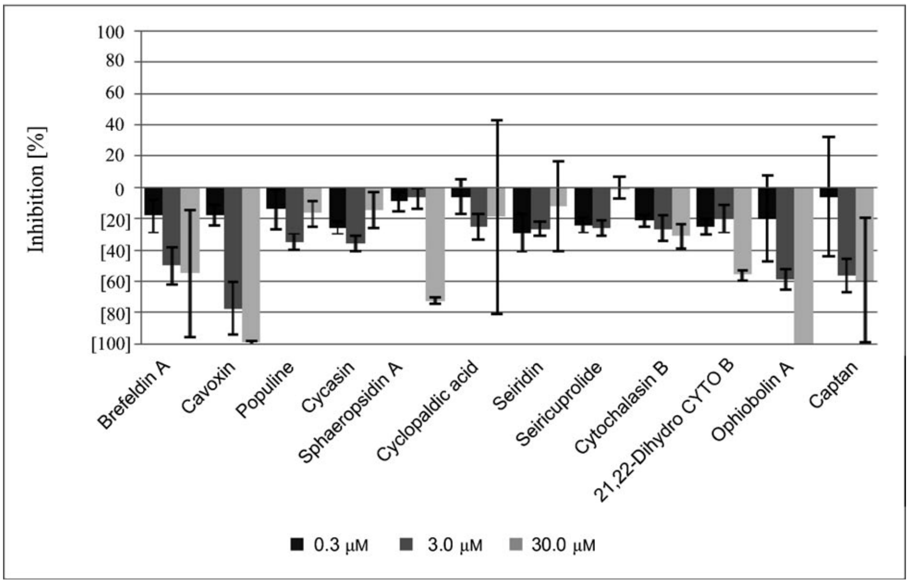


Fig. 1. *Mean fungal growth inhibition of microbial metabolite in a microdilution broth assay against* Phomopsis obscurans *at 144 h.* Captan was used as an internal commercial fungicide standard. Large deviations (as indicated by error bars) are likely due to the lack of synchronous growth by the test organism and/or reduced compound solubility in the aqueous culture media, especially when growth inhibition by a test compound was less at higher concentrations compared to lower concentrations.

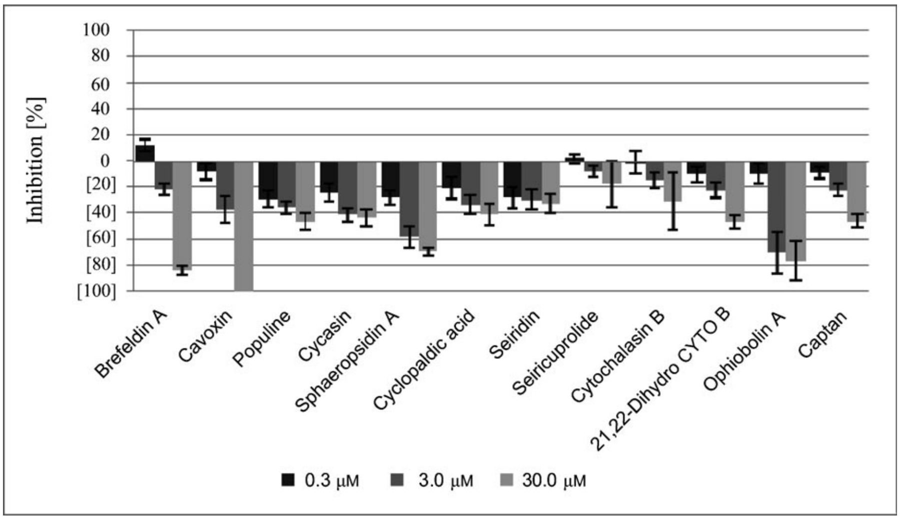


Fig. 2. *Mean fungal growth inhibition of each microbial metabolite in a microdilution broth assay against* Phomopsis viticola *at 144 h.* Captan was used as an internal commercial fungicide standard.

Fusicoccin (FC) was produced by *F. amygdali* as reported in [46]. The crystalline sample of FC, obtained as previously reported and preserved at $-20°$ under dark for *ca.* 26 years, showed by TLC (silica gel; $CHCl_3$/i-PrOH 9 : 1) and ^{1}H-NMR analyses the presence of some minor alteration products that are probably the well-known isomers formed by the shift of the Ac group from the C(3) to C(2) and C(4) of the glucosyl residue, resp., (*allo*- and *iso*-FC) [9][47]. Therefore, the sample was purified by a silica-gel column chromatography (CC; $CHCl_3$/i-PrOH 9 : 1). The FC derivatives (whose purity was ascertained by TLC and ^{1}H-NMR), triacetyl-8-oxo-FC [18], isomer of FC-deacetylaglycone [10], and 19-trityl-12-oxo-8,9-isopropylidene-FC-aglycone [19] were prepared starting from FC and following procedures as previously reported.

Sources of Test Compounds: Plant Metabolites and Their Derivatives. Papuline was also prepared by esterification of the (*S*)-2-hydroxy-3-phenylpropanoic acid. Cycasin was purified from the aq. extract of hulled seeds of *Cycas revolute* as reported in [5].

Lycorine [11] and ungeremine [20] were isolated from dried bulbs of *S. lutea* KER GAWL and *P. maritimun* L., resp., as reported by *Evidente* and co-workers. The purity of the samples was confirmed by TLC, ^{1}H-NMR, and optical-rotation analyses. 1-*O*-Acetyllycorine, 1,2-*O*,*O*-diacetyllycorine, 1,2-*O*,*O*-diacetyl-α-dihydrolycorine, lycorin-2-one, α-dihydrolycorine, and lycorine hydrochloride were prepared from lycorine according to the procedures of *Evidente* and co-workers [11][2]. *N*-Methyllycorine iodide and clivonine hydrochloride were generously supplied by Prof. *H. M. Fales*, Department of Health, Education and Welfare, Bethesda, MD, USA, and Prof. *C. Fuganti*, Istituto di Chimica, Politecnico di Milano, Italy, resp.

Algicide Bioassay. To evaluate the test compounds for their selective algicidal activities, procedures similar to those outlined by *Schrader et al.* [48] were applied. An isolate of *P. perornata* was obtained from a water sample collected from a Mississippi catfish pond [23]. An isolate of the green alga *Selenastrum capricornutum* PRINTZ (obtained from Dr. *J. C. Greene*, United States Environmental Protection Agency, Corvallis, Oregon) was used as a representative of green algae in the bioassay to determine selective toxicity of the test compounds. Each culture was maintained separately in continuous, steady-state growth using the conditions outlined in [48] to provide a source of cells growing at a constant rate.

Initially, the pure compounds listed in *Table 1* (*N*-methyllycorine iodide and α-dihydrolycorine were not evaluated because sample material was not available) were dissolved separately in 100% MeOH, except 1,2-*O*,*O′*-diacetyllycorine, clivonine chloride, and lycorine, which were dissolved in 100% CH_2Cl_2, MeOH/CH_2Cl_2 1 : 1, and MeOH/CH_2Cl_2 3 : 1, resp. Initial solns. were diluted to obtain concentrations of 2.0, 20.0, 200.0, and 2000.0 μM of each test compound, and diluted samples were added to empty wells (10 µl per well) of a 96-well microplate (Costar, Cambridge, Massachusetts). MeOH was allowed to evaporate completely from the microplate well before the addition of culture material (200 µl per well) of either *P. perornata* or *S. capricornutum*. Final test concentrations of each compound were 0.1, 1.0, 10.0, and 100.0 μM. Control wells contained only culture material. The same procedures for absorbance measurements and data management as described by *Schrader et al.* [48] were used. The lowest observed-effect concentration (*LOEC*) and lowest complete-inhibition concentration (*LCIC*) were determined for each test compound based upon graphing the absorbance measurement data. For the most active compounds (*i.e.*, selective and most toxic towards *P. perornata*), a 96-h IC_{50} (50% inhibition concentration) value was determined using the procedures of *Schrader et al.* [25].

Bactericide Bioassay. A culture of *E. ictaluri* (isolate S02-1039) was obtained from Mr. *Tim Santucci* (College of Veterinary Medicine, Mississippi State University, Stoneville, Mississippi), and cultures of two genotypes of *F. columnare* (*BioMed* (genomovar I) and *ALM-00-173* (genomovar II)) were obtained from Dr. *Covadonga Arias* (Department of Fisheries and Allied Aquacultures, Auburn University, Alabama). In addition, a culture of *Staphylococcus aureus* (ATCC #29213; methicillin-sensitive) was included in the bioassay as a representative test organism for *Gram*-pos. bacteria, and for comparison of results with the *Gram*-neg. bacteria *E. ictaluri* and *F. columnare*. To assure purity, cultures of *E. ictaluri* and *S. aureus* were maintained separately on 3.8% *Mueller–Hinton* (*MH*) agar plates (pH 7.3; *Becton, Dickinson and Company*, Sparks, Maryland), while cultures of *F. columnare* strains were maintained on modified *Shieh* agar plates (pH 7.2–7.4) [49]. Prior to conducting the bioassay, single colonies of the test cultures were used to prepare the assay culture materials as follows: *i*) for *E. ictaluri* and *S. aureus*, 45 ml of 3.8% *MH* at 0.5 *McFarland* standard [50] and *ii*) for *F. columnare*, each genotype was cultured separately in 75 ml of modified *Shieh* broth (18 h for *BioMed* and 24 h for *ALM-00-173*) at 28 ± 1° at 150 rpm on a rotary shaker (*Model C24KC*; *New Brunswick Scientific*, Edison, New Jersey).

Compounds were evaluated for antibacterial activity using a rapid 96-well microplate bioassay and according to the procedures of *Schrader* and *Harries* [50]. Florfenicol and oxytetracycline HCl, antibiotics used in medicated feed for catfish, were included as positive drug controls for each assay. In addition, control wells (no test compound added) were included in each assay. Technical-grade solvents were used to dissolve the test compounds, and all test compounds were dissolved in 100% MeOH, except 1,2-*O*,*O'*-diacetyllycorine, clivonine hydrochloride, and lycorine, which were dissolved in 100% CH_2Cl_2, MeOH/CH_2Cl_2 1:1, and MeOH/CH_2Cl_2 3:1, resp. Final concentrations of test compounds in the microplate wells were 0.01, 0.1, 1.0, 10.0, and 100.0 μM. Three replications were used for each dilution of each test compound and controls. Final results were converted to units of mg/l to allow comparison with previous studies.

Sterile 96-well polystyrene microplates (*Corning Costar Corp.*, Acton, Massachusetts) with flat-bottom wells were used to conduct the bioassay for compounds that were dissolved in 100% MeOH. To prevent solvent interaction with the polystyrene, sterile 96-well quartz microplates (*Hellma Cells, Inc.*, Forest Hills, New York) were used for compounds dissolved in CH_2Cl_2 and MeOH/CH_2Cl_2. Dissolved test compounds were added to microplate wells (10 μl/well). Solvents were allowed to completely evaporate before standardized bacterial culture (0.5 *MacFarland*) was added to the microplate wells (200 μl/well). Microplates were incubated at 29°. A *Packard* model *SpectraCount* microplate photometer (*Packard Instrument Company*, Meriden, Connecticut) was used to measure the absorbance (630 nm) of the wells at time 0 and 24 h, and the absorbance (570 nm) of each well was also recorded for the cell-viability portion of the assay (an additional 24 h of incubation) in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was added aseptically to the wells. The MTT had previously been dissolved in phosphate-buffered saline (pH 7.2; 5 mg/ml) and filter-sterilized (0.22 μm filter).

The means and standard deviations of absorbance measurements were calculated, graphed, and compared to controls to help determine the 24-h 50% inhibition concentration (IC_{50}) and 24-h minimum inhibitory concentration (*MIC*) values for each test compound (see [50]). The 24-h IC_{50} and *MIC* values for each compound tested were divided by the respective 24-h IC_{50} and *MIC* values obtained for the positive controls florfenicol and oxytetracycline to determine the relative-to-drug-control florfenicol (RDCF) and relative-to-drug-control oxytetracycline (RDCO) values.

Fungicide Bioassay. A direct bioautography assay and procedures of *Cantrell et al.* [51] were applied to evaluate compounds for antifungal activity against plant pathogenic fungi. Antifungal activities of pure compounds were evaluated against three *Colletotrichum* species at 4 and 8 μl of 20 mg/ml. Conidial suspensions of *Colletotrichum fragariae* and *C. acutatum* were adjusted to 3.0×10^5 conidia/ml with liquid potato-dextrose broth (PDB, *Difco*, Detroit, Michigan) and 0.1% *Tween-80*. Using a 50-ml chromatographic sprayer, each glass silica-gel TLC plate with fluorescent indicator (250 mm, *Silica Gel GF Uniplate, Analtech, Inc.*, Newark, Delaware) was sprayed lightly (to a dampness) three times with the conidial suspension. Inoculated plates were placed in a 30 × 13 × 7.5-cm moisture chamber (*398-C*, *Pioneer Plastics, Inc.*, Dixon, Kentucky) and incubated in a growth chamber at 24 ± 1° and 12-h photoperiod under 60 ± 5 μmol/m²/s light. Inhibition of fungal growth was measured for 4 d after treatment. Sensitivity of each fungal species to each test compound was determined by comparing size of inhibitory zones. Means and standard deviations of inhibitory zone size were used to evaluate antifungal activity of solvent fractions and pure compounds.

A standardized 96-well microdilution broth assay developed by *Wedge* and *Kuhajek* [52] was used to evaluate the antifungal activity of test compounds towards *Botrytis cinerea*, *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *Phomopsis viticola*, *P. obscurans*, and *Fusarium oxysporum.* Each microtiter test well received 80 μl of *RPMI 1640* (Roswell Park Memorial Institute mycological broth 1640, *Life Technologies*, Grand Island, New York) and 3-(morpholino)propanesulfonic acid (MOPS; *Sigma Chemical Co.*, St. Louis, Missouri) buffered broth (pH 7.0), 100 μl of conidial suspension at 1.0×10^4 conidia/ml, and 20 μl of test compound soln. The commercial fungicide captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose-response format using test compounds, with final treatment concentrations of 0.3, 3.0, and 30.0 μM. Microtiter plates (*Nunc MicroWell*, untreated; DK-Roskilde) were covered with a plastic lid and incubated in a growth chamber at $24 \pm 1°$ and a 12-h photoperiod under a light intensity of 60 ± 5 µmol/m^2/s. Growth was then evaluated by recording absorbance (620 nm) of each well using a microplate reader (*Model SpectraCount*; *Packard Instrument Company*, Meriden, Connecticut).

Using the 96-well plate micro-bioassay format, each chemical was evaluated in duplicate at three concentrations. Sixteen wells containing broth and inoculum served as positive controls, and eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. The experiments were repeated three times over time. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 and 72 h, except for *P. obscurans* and *P. viticola*, the data were recorded at 144 h. Analysis of variance of means for percent inhibition/stimulation of each fungal species at each dose of test compound relative to the untreated positive growth controls was used to evaluate fungal growth. Treatments were arranged as a split-plot design repeated four times. Whole-plots were fungal isolates and sub-plots were chemicals. Each dose level and response time was analyzed separately. The SAS system analysis of variance procedure (*Statistical Analysis System*, Cary, North Carolina) was used to identify significant factors, and *Fisher*'s protected LSD was used to separate means [53].

Herbicide Bioassay. The effect of test compounds on plant growth was determined with lettuce (*Lactuca sativa* L.) and bentgrass (*Agrostis stolonifera* L.) in 24-well plates according to the method of *Dayan et al.* [54]. A filter paper (*Whatman* No. 1) and 5 seeds of *L. sativa* or *ca.* 10 mg of *A. stolonifera* were placed in each well of a 24-well multiwell plate (type *CoStar 3524*; *Corning Inc.*, Corning, New York). Test compounds were dissolved in MeOH, CH_2Cl_2, or a mixture of MeOH and CH_2Cl_2, and test solns. were pipetted onto the filter paper and allowed to dry. To each test well, 200 µl of the double-deionized H_2O was added prior to seed addition. Plates were covered, sealed with parafilm, and held at 26° in a *Percival* growth chamber (model *CU-36L5*; *Percival Scientific, Inc.*, Boone, Iowa) under continuous flourescent light with an average intensity of 120 µmol/m^2/s. Phytotoxicity was qualitatively evaluated by visually comparing the amount of seed germination in each well with the untreated controls after 7 d. The qual. estimate of phytotoxicity was evaluated by using a rating scale of 0–5, where 0 = no effect and 5 = no growth or no germination of the seeds.

Termite Bioassay. To determine the effect of the test compounds on termites, termites were collected in bucket traps from four colonies of *Coptotermes formosanus* SHIRAKI in field sites in New Orleans, Louisiana [55]. The termites were maintained on spruce (*Picea* spp.) slats ($10 \times 4 \times 0.5$ cm) under conditions of *ca.* 100% relative humidity and 26°. Termites were identified using an identification key developed by *Scheffrahn* and *Su* [56].

Samples were tested separately by evenly blotting 100 µl of a soln. of each compound to be tested on a previously weighed disc of *Whatman No. 1* filter paper (2.5-cm diameter). The solvent, which was demonstrated to have no discernable effect on termite mortality or consumption as compared with H_2O, was allowed to evaporate from the filter paper over several hours, and the percentage of compound was defined as weight of compound per weight of the filter paper. The treated filter-paper discs were placed in plastic *Petri* plates (10×35 mm) and moistened with 100 µl of H_2O. For each treatment, 20 *C. formosanus* workers (3rd instar or greater as determined by size) and one soldier were placed in each *Petri* plate containing compound. Filter paper discs receiving only H_2O served as controls. All *Petri* plates were maintained at *ca.* 100% relative humidity and 26°. Treatments were replicated four times with termites for each replicate originating from a different *C. formosanus* colony.

Termite mortality was evaluated daily for 3 weeks. Consumption of test compound was determined by subtracting dried post-treatment from pre-treatment filter paper weights. Cumulative daily mortality and consumption (mean and standard deviation) were calculated for each treatment, and treatments were compared using ANOVA. Means were separated, following transformation to arcsine square root percent mortality [57], using a protected *Fisher* least-significant difference (LSD) test ($P < 0.05$). Actual mortality is reported in *Table 8*.

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REFERENCES

[1] A. M. Rimando, S. O. Duke, in 'Natural Products for Pest Management', Eds. A. M. Rimando, S. O. Duke, American Chemical Society, Washington, DC, USA, 2006, p. 2.
[2] A. Evidente, M. R. Cicala, G. Randazzo, R. Riccio, G. Calabrese, R. Liso, O. Arrigoni, *Phytochemistry* **1983**, *22*, 2192.
[3] M. Vurro, A. Evidente, A. Andolfi, M. C. Zonno, F. Giordano, A. Motta, *Plant Sci.* **1998**, *138*, 67.
[4] A. Evidente, G. Randazzo, N. S. Iacobellis, A. Bottalico, *J. Nat. Prod.* **1985**, *48*, 916.
[5] K. Nishida, A. Kobayashi, T. Nagahama, *Bull. Agric. Chem. Soc. Japan* **1955**, *19*, 77.
[6] A. Graniti, L. Sparapano, A. Evidente, *Plant Pathol.* **1992**, *41*, 563.
[7] A. Evidente, A. Andolfi, M. Vurro, M. C. Zonno, A. Motta, *Phytochemistry* **2002**, *60*, 45.
[8] A. Bottalico, R. Capasso, A. Evidente, G. Randazzo, M. Vurro, *Phytochemistry* **1990**, *29*, 93.
[9] A. Ballio, M. Brufani, C. G. Casinovi, S. Cerrini, W. Fedeli, R. Pellicciari, B. Santurbano, A. Vaciago, *Experientia* **1968**, *24*, 631.
[10] C. G. Casinovi, B. Santurbano, G. Conti, A. Malorni, G. Randazzo, *Gazz. Chim. Ital*. **1974**, *104*, 679.
[11] A. Evidente, I. Iasiello, G. Randazzo, *Chem. Ind.* **1984**, 348.
[12] A. Evidente, A. S. Kireev, A. R. Jenkins, A. E. Romero, W. F. A. Steelant, S. Van Slambrouk, A. Kornienko, *Planta Med.* **2009**, *75*, 501.
[13] A. Evidente, A. Andolfi, A. Cimmino, M. Vurro, M. Fracchiolla, R. Charudattan, *J. Agric. Food Chem.* **2006**, *54*, 1779.
[14] A. Evidente, N. S. Iacobellis, A. Scopa, G. Surico, *Phytochemistry* **1990**, *29*, 1491.
[15] A. Ballio, A. Evidente, A. Graniti, G. Randazzo, L. Sparapano, *Phytochemistry* **1988**, *27*, 3117.
[16] A. Evidente, G. Randazzo, A. Ballio, *J. Nat. Prod.* **1986**, *49*, 593.
[17] A. Evidente, L. Sparapano, A. Motta, F. Giordano, O. Fierro, S. Frisullo, *Phytochemistry* **1996**, *42*, 1541.
[18] A. Ballio, C. G. Casinovi, R. Capasso, A. Ferrara, G. Randazzo, *Gazz. Chim. Ital*. **1981**, *111*, 129.
[19] G. Randazzo, A. Evidente, R. Capasso, F. Colantuoni, L. Tuttobello, A. Ballio, *Gazz. Chim. Ital*. **1979**, *109*, 101.
[20] A. H. Abou-Donia, A.-A. Abib, A. S. El Din, A. Evidente, M. Gaber, A. Scopa, *Phytochemistry* **1992**, *31*, 2139.
[21] K. K. Schrader, F. E. Dayan, S. N. Allen, M. Q. de Regt, C. S. Tucker, R. N. Paul Jr., *Int. J. Plant Sci.* **2000**, *161*, 265.
[22] J. F. Martin, G. Izaguirre, P. Waterstrat, *Water Res.* **1991**, *25*, 1447.
[23] M. van der Ploeg, C. S. Tucker, C. E. Boyd, *Water Sci. Technol.* **1992**, *25*, 283.
[24] K. K. Schrader, M. E. Dennis, *Water Res.* **2005**, *39*, 2807.
[25] K. K. Schrader, M. Q. de Regt, P. D. Tidwell, C. S. Tucker, S. O. Duke, *Aquaculture* **1998**, *163*, 85.
[26] C. S. Tucker, A. T. Leard, 'Managing catfish off-flavors with diuron', Thad Cochran National Warmwater Aquaculture Center Fact sheet 003 (revised), Mississippi State University, Stoneville, Mississippi, 1999.
[27] P. C. Leung, W. A. Taylor, J. H. Wang, C. L. Tipton, *J. Biol. Chem.* **1984**, *259*, 2742.
[28] G. W. Kerson, J. A. Miernyk, K. Budd, *Plant Physiol.* **1984**, *75*, 222.
[29] A. Pettersson, B. Bergman, *Microbiol. Lett.* **1989**, *60*, 95.
[30] L. A. Onek, P. J. Lea, R. J. Smith, *Arch. Microbiol.* **1994**, *161*, 352.
[31] T. Fujiwara, K. Oda, S. Yokota, A. Takatsuki, Y. Ikehara, *J. Biol. Chem.* **1988**, *263*, 18545.
[32] P. Klesius, J. Evans, C. Shoemaker, *Aquacult. Health Int.* **2006**, *4*, 20.
[33] J. A. Plumb, ' Health Maintenance and Principal Microbial Diseases of Cultured Fishes', Iowa State University Press, Ames, 1999, pp. 328.
[34] C. E. Boyd, C. S. Tucker, 'Pond Aquaculture Water Quality Management', Kluwer Academic Publishers, Boston, 1998, pp. 700.
[35] C. A. Shoemaker, O. Olivares-Fuster, C. R. Arias, P. H. Klesius, *Vet. Microbiol.* **2008**, *127*, 353.
[36] M. Normatov, Kh. A. Abduazimov, S. Yu. Yunusov, *Uzbeksk. Khim. Zh.* **1965**, *9*, 25.
[37] A. A. Ali, H. M. El Sayed, O. M. Abdalliah, W. Steglich, *Phytochemistry* **1986**, *25*, 2399.
[38] S. Ghosal, Y. Kumar, S. K. Singh, A. Kumar, *J. Chem. Res. Synop.* **1986**, 112.

[39] S. Ghosal, S. K. Singh, R. S. Srivastava, *Phytochemistry* **1986**, *25*, 1975.
[40] G. R. Pettit, Y. Meng, D. L. Herald, J. C. Knight, J. F. Day, *J. Nat. Prod.* **2005**, *68*, 1256.
[41] S. Ghosal, S. K. Singh, Y. Kumar, S. Unnikrishnan, S. Chattopadhyay, *Planta Med.* **1988**, *54*, 114.
[42] A. Evidente, G. Randazzo, G. Surico, P. Lavermicocca, O. Arrigoni, *J. Nat. Prod.* **1985**, *48*, 564.
[43] Z. Iqbal, H. Nasir, S. Hiradate, Y. Fujii, *Weed Biol. Manag.* **2006**, *6*, 221.
[44] A. Evidente, A. Motta, *Stud. Nat. Prod. Chem*. **2002**, *26G*, 581.
[45] A. Berestetskiy, A. Dmitriev, G. Mitina, I. Lisker, A. Andolfi, A. Evidente, *Phytochemistry* **2008**, *69*, 953.
[46] A. Ballio, A. Carilli, B. Santurbano, L. Tuttobello, *Ann. Ist. Super. Sanità* **1968**, *4*, 317.
[47] A. Ballio, C. G. Casinovi, M. Framondino, G. Grandolini, G. Randazzo, C. Rossi, *Experientia* **1972**, *28*, 1150.
[48] K. K. Schrader, M. Q. de Regt, C. S. Tucker, S. O. Duke, *Weed Technol.* **1997**, *11*, 767.
[49] A. Decostere, R. Ducatelle, F. Haesebrouck, *Vet. Res.* **2002**, *150*, 694.
[50] K. K. Schrader, M. D. Harries, *Aquacult. Res.* **2006**, *37*, 928.
[51] C. L. Cantrell, B. P. Case, E. E. Mena, T. M. Kniffin, S. O. Duke, D. E. Wedge, *J. Agric. Food Chem.* **2008**, *56*, 5062.
[52] D. E. Wedge, J. M. Kuhajek, *SAAS Bull. Biochem. Biotech.* **1998**, *11*, 1.
[53] R. G. D. Steel, J. H. Torrie, 'Principles and Procedures of Statistics: A Biometrical Approach', 2nd edn., McGraw Hill Book Company, New York, 1980, p. 176.
[54] F. E. Dayan, J. G. Romagni, S. O. Duke, *J. Chem. Ecol.* **2000**, *26*, 2079.
[55] N. Y. Su, R. H. Scheffrahn, *Sociobiology* **1986**, *12*, 299.
[56] R. H. Scheffrahn, N. Y. Su, *Fla. Entomol.* **1994**, *77*, 460.
[57] SAS 1990, SAS Institute, Cary, North Carolina, Vol. 6.

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